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A Comparative Study of Carotid Atherosclerotic Plaque Microvessel Density and Angiogenic Growth Factor Expression in Symptomatic Versus Asymptomatic Patients

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Abstract *Objective:* A challenge facing clinicians is identifying patients with asymptomatic carotid disease at risk of plaque instability. We hypothesise that locally released angiogenic growth factors contribute to plaque instability.
Methods: Carotid endarterectomy specimens from eight symptomatic and eight asymptomatic patients were interrogated for microvessel density and angiogenic growth factor expression histologically using immunofluorescence, and biochemically using quantitative real-time polymerase chain reaction (q-RT-PCR). Bio-Plex™ suspension array was used to assess circulating biomarkers in venous blood from the same patients and six healthy age-matched controls.
Results: Immunofluorescence demonstrated significantly greater neovessel density in symptomatic plaques ($P = 0.010$) with elevated expression of hepatocyte growth factor (*HGF*) ($P = 0.001$) and its receptor *MET* ($P = 0.011$) than in asymptomatic plaques. The q-RT-PCR demonstrated up-regulation of *Endoglin* (CD105), *HGF* ($P = 0.001$) and *MET* ($P = 0.011$) in the plaques of symptomatic versus asymptomatic patients. Bio-Plex™ suspension array demonstrated elevated HGF ($P = 0.002$) serum levels in symptomatic versus asymptomatic patients and healthy controls, and decreased platelet-derived growth factor (PDGF) ($P = 0.036$) serum levels in symptomatic versus asymptomatic patients.

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Conclusion: Plaque instability may be mediated by HGF-induced formation of new microvessels, and decreased vessel stability resulting from decreased PDGF. Suspension array technology has the potential to identify circulating biomarkers that correlate with plaque rupture risk.

Stroke is the third most common cause of death in the Western world and a major economic and resource burden.[1] An important cause of stroke is the transformation of a quiescent carotid artery atherosclerotic lesion into an unstable morphology, which becomes vulnerable to rupture and thrombo-embolism.

Carotid endarterectomy is established for preventing future strokes in patients with symptomatic and, to a lesser extent, asymptomatic disease.[2] A challenge facing vascular specialists is differentiating patients with asymptomatic carotid disease who are at risk from plaque instability. Identifying such patients would reduce the number of unnecessary surgical procedures being performed. However, to date, there are no clinically validated biomarkers to predict asymptomatic patients at the risk of stroke. Identification of biomarker signatures associated with plaque instability may allow for a more precise patient-selection strategy for carotid surgery, in the presence of significant but asymptomatic disease.

An important component of atherogenesis is the formation of new microvessels within the diseased vessel wall, a process known as neovascularisation. A multitude of pro-angiogenic factors[2] has been implicated in this process, including hepatocyte growth factor (*HGF*).[3] HGF, a mesenchyme-derived pleiotrophic cytokine initially recognised as a potent mitogen for hepatocytes,[4] is now acknowledged to have strong mitogenic and morphogenic effects on various cell types, including endothelial cells (ECs) and smooth muscle cells (SMCs).[5–7] *HGF* functions by binding to its corresponding receptor, *MET*, a member of the tyrosine kinase family.

This study investigates the hypothesis that symptomatic carotid plaques are associated with increased plaque microvessel density as compared with asymptomatic lesions. We postulate that these microvessels are correlated to localised expression and systemic levels of pro-angiogenic growth factors and associated receptors, in particular, *HGF* and *MET*. The aims of the study are to (1) identify and quantify microvessels in human atherosclerotic plaques obtained from symptomatic versus asymptomatic patients undergoing carotid endarterectomy; (2) establish the levels of gene expression of *HGF* and *MET*; and (3) elucidate whether *HGF* or any other angiogenic growth factors are differentially modulated in symptomatic or asymptomatic disease, thus lending credence to their suitability as potential biomarkers in human serum, which may identify patients at risk from plaque instability.

Materials and methods

Tissue collection and sectioning

Carotid plaques from eight symptomatic and eight asymptomatic patients were analysed for this preliminary study. For Bio-Plex™ analysis, serum from these patients was compared against that of six healthy age-matched controls.

Local Research Ethics Committee approval was granted for the use of human carotid plaques and serum for this study (08/H1014/88). Carotid atherosclerosis specimens were obtained from patients undergoing carotid endarterectomy for $\geq$70% internal carotid artery stenoses. Prior to surgery, patients were consented and assessed with respect to age, sex, co-morbidity and symptomatology (Table 1). All patients enrolled on this study were on anti-platelet and HMG-CoA reductase inhibitor therapy. The surgical procedures were performed using a standardised technique under general anaesthesia.

All plaques were processed at the time of surgery. After removal, the plaque was divided into three sections; one washed in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde and processed for paraffin embedding, the second fixed in OCT embedding medium and frozen at $-80\,^{\circ}C$ and the third put into RNALater® (Applied Biosystems, Foster City, CA, USA) for RNA analysis. Formalin-fixed, paraffin-embedded sections were used to confirm the gross morphology of the plaque by preparing 7 μm haematoxylin and eosin-stained sections. A panel of archived internal mammary arterial sections was used as non-atherosclerotic controls, which were obtained from arterial segments surplus to requirements from coronary artery bypass graft (CABG) surgery and previously described.[8]

Quantitative real-time polymerase chain reaction (q-RT-PCR)

RNA extraction and cDNA synthesis

RNA extraction was carried out by homogenisation of the tissue sample using a Hybaid RiboLyser™ Cell Disrupter. Tissue was dissolved in 1 ml of TRIzol (Invitrogen,

Table 1 Patient demographics.

Risk factor/group	Symptomatic ($n = 8$)	Asymptomatic ($n = 8$)
Age, median (range)	66 (61–71)	68 (67–76)
Sex, male: female	6:2	5:3
Smoking, *n* (%)	7 (88%)	5 (63%)
Statin use, *n* (%)	8 (100%)	8 (100%)
Symptoms – TIA, *n* (%)	7 (88%)	–
Amaurosis fugax, *n* (%)	1 (12%)	–
Diabetes, *n* (%)	5 (63%)	4 (50%)

All values $P > 0.05$ between both groups, except the variable group (patient symptomatology).

Carlsbad, CA, USA) per 50 mg of tissue in a lysing matrix bead tube (MP Biomedicals Cat # 6913-100, Solon, OH, USA). The tissue solution was ribolysed for two cycles of 20 s. The homogenised sample was incubated for 5 min at 22 °C to permit complete dissociation of nucleoprotein complexes. Then, 0.2 ml of choloform per 1 ml of TRIzol was added, centrifuged at 12 000 × g for 15 min at 5 °C. Following the centrifugation, the mixture separated and the aqueous colourless phase containing the RNA was transferred to a fresh tube, mixed with 0.5 ml of isopropyl alcohol and centrifuged at 12 000 × g for 10 min at 5 °C. The supernatant was removed, preserving the RNA pellet, which was washed with 75% ethanol, centrifuged at 7500 × g for 5 min at 5 °C. The resulting RNA pellet was air-dried for 5 min and re-dissolved in 20 μl of RNAase-free water, with subsequent DNAase treatment.

Complementary DNA (cDNA) was synthesised from 1 μg of total RNA using a reaction mix with a final volume of 20 μl: 4 μl of 5× buffer, 1 μl of oligo(dT)$_{15}$ primer (100 pmol μl^{-1}), 2 μl of dNTP (10 mM), 0.5 μl of RNAase inhibitor, 0.4 μl of AMV RT enzyme (Roche, Basel, Switzerland) and diethylpyrocarbonate (DEPC)-treated H_2O adjusted to the volume of 20 μl. The reaction was performed at 42 °C for 1 h to produce cDNA.

Q-RT-PCR

Confirmation that the samples expressed *Endoglin* (CD105), *HGF* and *MET* was achieved using SYBR Green real-time PCR. PCR primer sequences were designed using design software (Primer Express™ ver. 2.0.0, Applied Biosystems) (Table 2). BLAST (Basic Local Alignment Search Tool) searches were performed for all primer sequences to confirm gene specificity. The q-RT-PCR assays were performed using the 7900HT Fast Real-Time PCR System (Applied Biosytems) in a 96-well plate format. Real-time data were analysed using the Sequence Detection System 2.2.1 software (v20040907-2, 2004, Applied Biosystems, Foster City, CA, USA) with the detection threshold set manually at 0.05 for all the assays. All transcripts were standardised to Actb (β-actin) (housekeeping gene). Quantification of relative gene expression was calculated by the comparative Ct method (2−ΔΔCt).[9]

Immunohistochemistry and immunofluorescence

Immunohistochemistry was used for primary antibody staining. Immunofluorescence was used for endothelial cell localisation and quantification. For immunohistochemistry, frozen sections were cut at 6 μm on the cryostat and fixed in pre-cooled 100% acetone for 10 min. The serial sections were permeabilised using 0.1% Triton x-100 in PBS. Endogenous peroxide activity was blocked using 0.3% hydrogen peroxide in methanol for 20 min at 4 °C. The blocking serum was applied to each sample (100 μl) and was kept at 22 °C for 1 h. A negative control was set up for each slide (the sample was treated with non-immune, non-specific antibodies), primary antibody *HGFα*, (1:50, H-145 sc-7949, Lot #L0804, Santa Cruz Biotechnology, Santa Cruz, CA, USA), *MET C-28*, (1:20, sc-161, Lot #H2206, Santa Cruz Biotechnology), *CD31*, (1:30, Cat No M0823, DakoCytomation, Glostrup, Denmark), smooth muscle *α-Actin*, (1:400 A5228, DakoCytomation) was placed on the sample for 1 h at 22 °C. The samples were then washed in PBS, and incubated for 1 h at 22 °C with the corresponding horseradish peroxidise (HRP)-conjugated secondary antibody. The samples were then treated with 3,3′-diaminobenzidine tablets (Sigma Fast™ D-4168, DAB peroxidise substrate, Sigma–Aldrich Co., USA) until a colour change was noted. The reaction was then stopped, slides were washed in PBS, counterstained with Mayer's haematoxylin (H) and mounted with mounting medium (Vector Laboratories, Burlingame, CA, USA).

For immunofluorescence, serial frozen sections were exposed to 5 mg ml^{-1} fluorescein-labelled UEA-I (Ulex Europaeus Agglutinin-1) (Vector Laboratories, Burlingame, CA, USA) diluted 1:50 in water for 2 min. The sections were rinsed in PBS for 2 min, sequentially dehydrated (70% ethanol for 30 s, 95% ethanol for 30 s and 100% ethanol for 1 min). Sections were counterstained with 4′–6′diamidino-2-phenylindole (DAPI) to localise the nuclei of all cell types, adding further distinction to any positively stained microvessels. The slides were air-dried for 2 min and immediately mounted using VECTASHIELD® fluorescence mounting media (H-1000, 10 ml, Vector Laboratories, Burlingame, CA, USA). All slides were viewed under a Leica colour/fluorescent microscope (DFC320/DFC 350FX). Images were captured

Table 2 Primers for quantitative RT-PCR.

Gene of interest	Accession number	Primer position[a]	Forward primers (5′ → 3′)	Reverse primers (5′ → 3′)
HGF	NM_000601	F982 R1032	ACTCTTGACCCTCACACCCG	AGCGCATGTTTTAATTGCACAG
MET	NM_000245	F5006 R5056	CACCATCCCCGGCTAATTT	ATGGCAAAACCCCGTCTCTA
Endoglin	NM_000118	F1530 R1580	TGTGAGGCAGAGGACAGGG	GGAGTAAGCACTGCGCAAGAC
ACTB	NM_001101	F3255 R3305	GGACATGGAGAAGATGTG	CATCTCCTGCTCGAAGTC

Forward and reverse primers for PCRs. BLAST (Basic Local Alignment Search Tool) searches were performed for all primer sequences to confirm gene specificity. All primers were synthesised from SIGMA–ALDRICH Company Ltd., Dorset, UK.

[a] Refers to position from the transcription start site within the gene sequence. F&R refers to forward and reverse orientation respectively.

using Leica Application Suite Version 2.8.1 (Build:1554, 2003–2007, Leica Microsystems, Switzerland, Ltd., CMS GmbH). Images using different fluorescent wavelengths were then merged.

Bio-plex suspension array

The Bio-Plex™/Luminex Bead array (Bio-Rad Laboratories Ltd., CA, USA) was used to analyse serum concentration of angiogenic factors in patients' venous blood including angiopoietin 2, follistatin, granulocyte-colony stimulating factor (GCSF), HGF, interleukin (IL)-8, leptin, platelet-derived growth factor-BB (PDGF-BB), PCAM1 and vascular endothelial growth factor (VEGF) (Bio-Rad's 'Pro-Human Angiogenic 9-plex plate' Cat No: #171A4S11M).

Collection of serum samples

The 10 ml of venous blood withdrawn aseptically into sterile heparinised tubes was taken preoperatively and centrifuged at 3200 rpm for 15 min at 4 °C. The resulting clear serum was stored at −20 °C in aliquots until needed for analysis.

Bio-Plex™ luminex immunoassay

Levels of nine angiogenic analytes, including HGF, were analysed in patient serum using Bio-Plex™ immunoassay (Bio-Rad) according to the manufacturer's instructions. A standard dilution series was made. All samples were compared against normal human serum samples (control).

Bio-Plex Pro™ angiogenesis data acquisition

Data were analysed using Bio-Plex™ Manager software version 5.0. The plate format included bead region data for each analyte, as well as dilution factors for both the controls and standards. Data acquisition was specified for 100 beads per region. The bead map was set to 25 per region, and the sample size was set to 50 μl. Default DD gate values were set to 5000 (low) and 32 000 (high).

Statistical analysis

The Mann–Whitney *U*-test was used to compare the groups. This non-parametric method was used because the measurements of the angiogenic factors were not normally distributed. Values of $P < 0.05$ were considered significant (SPSS Inc., v16.0, Chicago, IL, USA).

Results

Immunohistochemistry analysis

Microvessel density quantification

The average carotid cross-sectional area for both symptomatic and asymptomatic plaques was 15 mm^2 and 10 sections from each block were examined. Fig. 1 shows a microvessel at high power (×40). Microvessels were counted from five random fields of view (2.25 mm^2 per section) and 45 mm^2 was examined within each plaque and the average was calculated. The median neovessel density (vessels mm^{-2}) was higher in symptomatic plaques than asymptomatic plaques (8 (range 3–16) vs. 3 (range 0–7); $P = 0.010$) plaques (Fig. 2).

Localisation of α-SMA

Neovessels themselves can often be characterised as 'mature' or 'immature'. The latter have not acquired the coating of smooth muscle cells associated with mature vessels. Immunostaining of *α-SMA* revealed a coating of some microvessels in the plaques, in addition to the presence of irregular, diffuse staining (Fig. 3). These smooth muscle cells appeared to have lost their morphological integrity that occurs within a normal medial layer.

Molecular analysis of HGF/MET expression

Modulation of gene expression within plaque specimens

The q-RT-PCR analysis of *HGF* and *MET* revealed a 4.7- and 2.8-fold mRNA expression increase, respectively, in symptomatic versus asymptomatic plaques ($P = 0.001$, $P = 0.011$, respectively, Fig. 4). Although there was 1.8-fold increase in *Endoglin* mRNA expression in symptomatic versus asymptomatic plaques, this did not reach statistical significance ($P = 0.184$).

Localisation of HGF/MET protein

Localisation of HGF protein

Using *HGF* antibodies, immunohistochemistry was performed on both cryosections and paraffin-fixed sections of human carotid atherosclerotic plaques. Some of the regions within the plaques contained endothelial cells that were positive for HGF immunoreactivity. This was achieved by

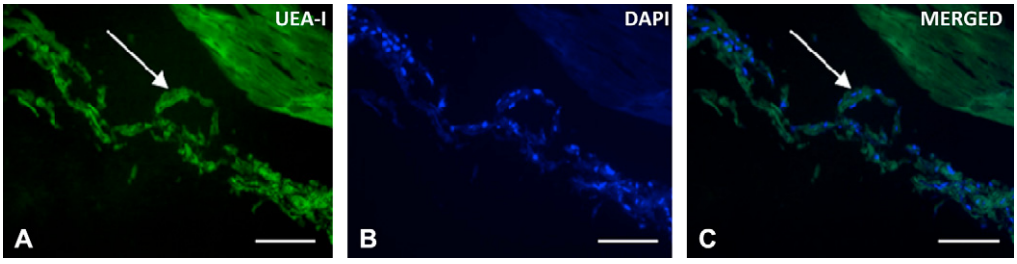

Figure 1 Characterisation of microvessels in carotid plaques (UEA-I) Representative micrograph showing identification of microvessels from carotid samples using fluorescein-labelled UEA-I (Ulex Europaeus Agglutinin-I) staining. The endothelial cells stained green enabling identification of microvessels (white arrows A & C). Microvessels were distinguished from background luminescence by counterstaining sections with DAPI which detected nuclear staining of all cells in the vessel wall (B). Images are merged in (C) (Bar = 50 μm).

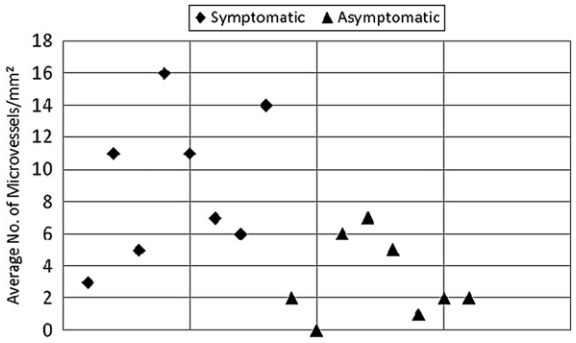

Figure 2 Number of microvessels identified from plaques of symptomatic and asymptomatic patients. Microvessels were identified in 5 fields of view within each section. Ten sections were quantified from each patient block. 8 symptomatic plaques were compared against 8 asymptomatic plaques. Statistical analysis using Mann–Whitney *U*-test ($P = 0.01$).

serial sections co-staining with *HGF* and the endothelial cell marker, *CD31* (Fig. 5). *HGF*-positive stains were found consistently in all sections. However, *HGF* staining was not detected in the non-atherosclerotic internal mammary arterial sections.[10]

Localisation of MET

Immunostaining for MET was positive in both symptomatic and asymptomatic plaques. The MET positive cells also appear to demonstrate α-smooth muscle positive staining. This supports and extends our previous findings, where we demonstrated the presence of MET in human tibial arterial atherosclerotic lesions.[11]

Analysis of potential serum markers of disease

Serum angiogenesis factors in symptomatic versus asymptomatic patients

The serum concentration of nine angiogenic growth factors was evaluated (Fig. 6): angiopoietin 2, follistatin, GCSF, HGF, IL-8, leptin, PDGF-BB, PECAM1 and VEGF. HGF showed a statistically significant elevation in the serum of subjects with symptomatic carotid disease than in their asymptomatic counterparts and healthy controls (HGF: 1033 pg ml^{-1} (952–2538) vs. 78.9 pg ml^{-1} (46.5–113), respectively; $p = 0.002$), while PDGF (PDGF-BB: 531 pg ml^{-1} (429–655) vs. 1097 pg ml^{-1} (986–1674); $p = 0.036$) was significantly reduced in symptomatic when compared with asymptomatic patients. There was no statistical significance with the other angiogenic factors in the serum of subjects with symptomatic carotid disease compared with their asymptomatic counterparts (Table 3).

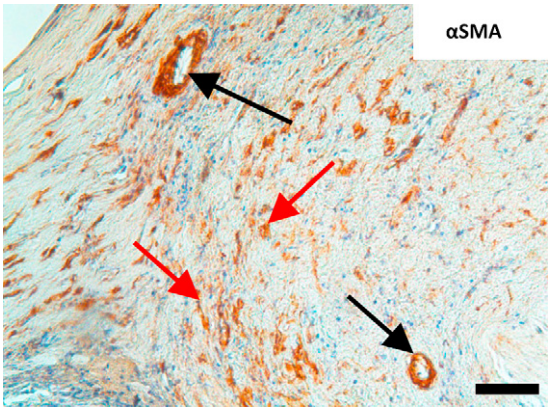

Figure 3 Characterisation of smooth muscle actin in carotid plaques (αSMA) Morphology of smooth muscle actin coverage within human carotid atherosclerotic plaques. In addition to clear microvessel presence (black arrows), there was also diffuse, poorly organised positive smooth muscle cell staining (red arrows) (Bar = 50 μm).

Discussion

Several studies have linked serum levels of HGF with vascular disease, and we have previously reported MET positive SMCs in human tibial artery atherosclerotic lesions.[11] This study has localised and quantified *HGF* gene expression in symptomatic and asymptomatic human carotid plaques by molecular techniques. Given that HGF regulates endothelial cell growth, apoptosis and angiogenesis, its presence in plaques could contribute to the pathogenesis of atherosclerosis.

In this preliminary study, the aim was to determine whether changes in systemic levels and local expression of *HGF* could correlate to patient symptomatology. This was achieved using a number of approaches: localisation of this pro-angiogenic growth factor by standard immunohistochemistry techniques, a quantitative analysis by q-RT-PCR and a systemic protein serum screen by Bio-Plex™ suspension array technology. Our findings have shown the localisation of HGF and its biological receptor, MET, in carotid plaques. The q-RT-PCR analysis has revealed a significantly higher expression of *HGF* in symptomatic versus asymptomatic patients. Bio-Plex™ suspension array has also confirmed an increase in serum HGF levels, in addition to the elevated localised expression amongst the symptomatic cohort.

Angiogenesis has been shown in symptomatic carotid plaques. McCarthy et al.[12] and Mofidi et al.[13] demonstrated a strong association between an increased number of new vessels within symptomatic carotid plaques and stroke. It was postulated that plaque angiogenesis may be significant in the development of symptomatology. The present study supports this observation, whilst at the same time reveals that the increased neovascularisation, in symptomatic plaques, is associated with increased *HGF* and *MET* gene expression.

The increased microvessels in the plaque of unstable lesions could act as a source of activated endothelial cells; cells that are vital for leucocyte adhesion and activation.[13] Infiltration of leucocytes and other inflammatory cells results in release of proteases, most notably of the matrix metalloproteinase family, which cause plaque disruption.[13] The morphological nature of the 'immature' neovessels may lend themselves to rupture more easily than their mature and stable counterparts, creating an escalating pro-inflammatory microenvironment.

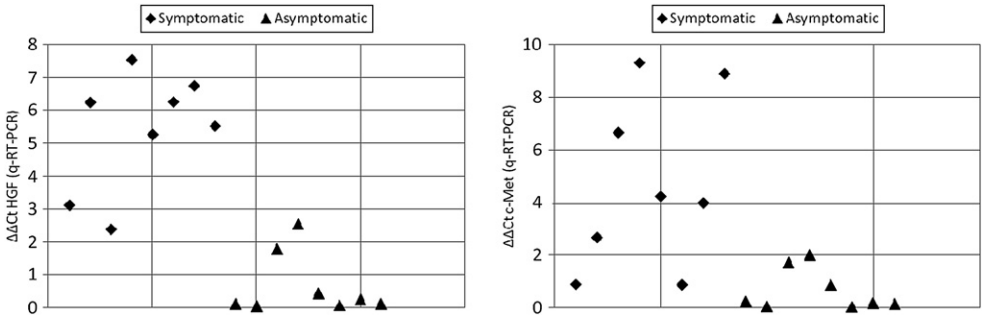

Figure 4 HGF and c-Met mRNA expressions detected by q-RT-PCR. Statistical analysis compares ΔΔCt values, for each gene of interest. 8 symptomatic plaques were compared against 8 asymptomatic plaques. Statistical analysis using Mann—Whitney *U*-test. HGF ($P = 0.001$) c-Met ($P = 0.011$).

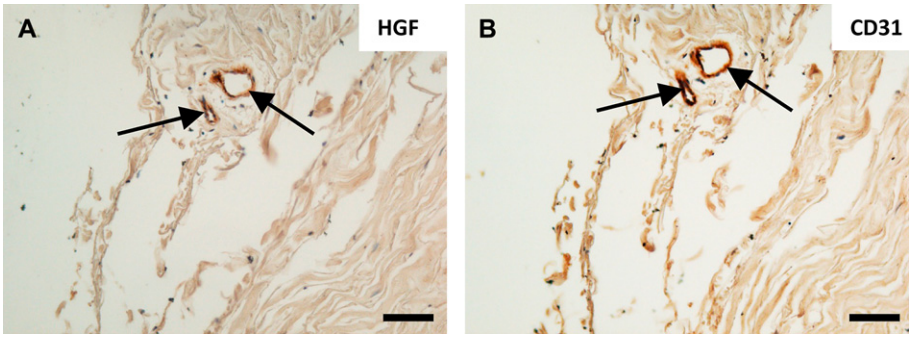

Figure 5 Localisation of HGF within human carotid plaques HGF was characterised within microvessels (black arrows - left). This was achieved by serial section staining (right), using CD31 as an endothelial cell marker. Sections were counterstained with Haematoxylin (Bar = 50 μm).

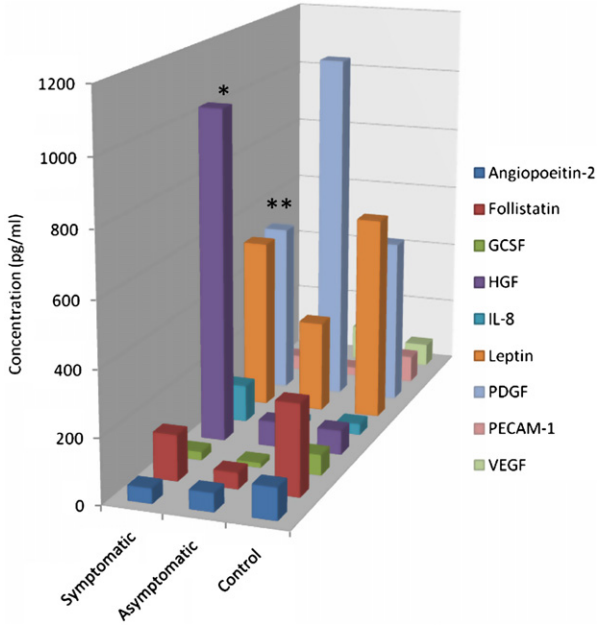

Figure 6 | Bio-Plex™ angiogenesis serum analyte levels in symptomatic, asymptomatic and healthy control patients. Serum harvested from patients and subjected to Bio-Plex suspension array analysis. 8 symptomatic and 8 asymtpomatic patients were compared against 6 healthy matched controls. Statistical analysis using Mann—Whitney *U*-test. HGF * ($P = 0.002$). PDGF ** ($P = 0.036$).

Serum levels of PDGF

Analysis of patient serum did reveal a significantly lower level of PDGF in symptomatic compared with asymptomatic patients. Angiogenesis not only depends on endothelial cell invasion and proliferation; it also requires mural cell coverage of vascular sprouts for vessel stabilisation.[14] These processes are coordinated by many factors, including PDGF and its receptor, which affects vascular smooth muscle cells (VSMCs). After PDGF receptor blockade in mouse models, Greenberg et al.[15] observed suppression of angiogenesis in the absence of PDGF, consistent with a role of PDGF in the release of pro-angiogenic factors and the lack of mural cell recruitment.[16] Greenberg et al. go on to comment that exclusive PDGF treatment results in a robust influx of alpha-smooth muscle actin-expressing cells being recruited to the surface of patent blood vessels.[17]

In light of these studies, one can postulate that the decreased levels of PDGF in symptomatic patients when compared with asymptomatic patients may play a role in vessel stabilisation. The lower levels in symptomatic patients may impair or restrict the extent of SMA recruitment to the neovessels in the plaque, leading to an increased number of 'leaky' vessels, and more extensive plaque instability. However, serum levels of PDGF in symptomatic subjects are similar to levels of PDGF in the healthy controls; there was no significant difference

Table 3 Serum angiogenesis factors in symptomatic versus asymptomatic patients.

Analyte	Symptomatic — pg/ml (range)	Asymptomatic — pg/ml (range)	*P* value
Angiopoeitin-2	47.1 (38.1—73.5)	58.1 (35.8—67.4)	0.770
Follistatin	143 (195—309)	51.5 (43.8—165)	0.078
GCSF	26.6 (19.5—75.8)	15.4 (11.4—40.8)	0.770
HGF	1033 (952—2538)	78.9 (46.5—113)	0.002*
IL-8	116 (70.5—212)	19.5 (12.4—74.9)	0.599
Leptin	529 (471—654)	286 (257—346)	0.262
VEGF	48.6 (14.8—54.5)	112 (94.6—196)	0.133
PECAM-1	46.2 (36.4—79.7)	26.6 (15.4—97.1)	0.170
PDGF	531 (429—655)	1097 (986—1674)	0.036*

Serum harvested from patients and subjected to Bio-Plex suspension array analysis. Values are median, the range in parenthesis. Statistical analysis using Mann—Whitney *U*-test. Significant values are indicated by *.

between these two groups. Levels of PDGF may, therefore, be mediated by more complex mechanisms and play a more influential role in the diseased state.

HGF in the atherosclerotic plaque

The data in this study demonstrate the presence of HGF in atherosclerotic lesions compared with no detection in the non-atherosclerotic controls, suggesting that HGF may participate in this disease process.

The invasiveness of monocytes is stimulated by HGF treatment, implicating a role for HGF in monocyte recruitment.[18] Furthermore, interactions between VSMCs and monocytes result in HGF production from both cell types, which could perpetuate the inflammatory response.[19] As HGF is a potent angiogenic factor, its presence in atherosclerotic lesions could aid neovascularisation within the plaque. The data presented demonstrate HGF staining in association with neovascularised areas within the plaque. Angiogenesis and migration of endothelial cells through the extracellular matrix requires degradation of the matrix, which can be accomplished by matrix metalloproteinases that also can be induced by HGF.[20]

Clinical impact

In the context of carotid atherosclerotic plaques, perhaps a more profound understanding of plaque stability will help ensure an improved and specific patient-selection strategy for interventional therapies. Suspension array technology has great potential for the identification of circulating biomarkers that may correlate with angiogenesis and plaque rupture risk. HGF itself may even be used as a biomarker to screen asymptomatic patients with moderate carotid disease. Another possible target is to question whether the plaque stabilisation could be achieved by local therapies. Local delivery of anti-angiogenic agents has met with recent clinical success in the treatment of age-related macular degeneration.[21] To limit potential systemic side-effects of any therapeutic agents, stent- or catheter-based therapies may be an effective means of localised drug delivery to eliminate neovascularisation in a clinical setting. A recent experimental study[22] with arterial atherosclerotic plaques has shown that stent-based delivery of anti-angiogenic agents may be a viable treatment option.

Limitations of the study

Although the data presented have proposed potentially novel screening biomarkers for carotid disease, the study itself has limitations. Patient numbers were small and should be considered a limiting factor in the analysis. It should, therefore, be emphasised that this study is preliminary in nature. In addition to this, future studies should also correlate serum biomarker levels with the degree of symptoms and samples should also be taken postoperatively.

Conclusion

These data show a significantly increased microvessel density and expression of *HGF* and *MET* in symptomatic plaques in comparison to their asymptomatic counterparts. The transition from a stable to an active plaque may thus be partly mediated by an increased expression of these angiogenic factors.

Expression of *HGF* in the carotid plaques amongst symptomatic patients, with q-RT-PCR, was correlated with increased systemic HGF levels in these patients. Suspension array analysis revealed a significantly higher concentration of HGF in symptomatic patients, when compared with asymptomatic and healthy controls. Levels of PDGF were found to be significantly lower in symptomatic than in asymptomatic patients.

The evidence provided suggests that HGF appears to promote the progression of carotid atherosclerotic plaques. However, the presence of cytokines and many other growth factors may affect the endothelial response to HGF. This study may ultimately indicate new interventional therapies for the prevention of plaque formation, possibly by targeting neovascularisation via the *HGF/MET* signalling pathway.

Conflict of interest

None.

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